

The present invention also contemplates the use of nucleases isolated from a
organisms that grow under a variety of conditions. The genes for the FEN-1/XPG
class of enzymes are found in organisms ranging from bacteriophage to humans to the
extreme thermophiles of Kingdom Archaea. For assays in which high temperature is
5 to be used, it is contemplated that enzymes isolated from extreme thermophiles may
exhibit the thermostability required of such an assay. For assays in which it might be
desirable to have peak enzyme activity at moderate temperature or in which it might
be desirable to destroy the enzyme with elevated temperature, those enzymes from
organisms that favor moderate temperatures for growth may be of particular value.

10 An alignment of a collection of FEN-1 proteins sequenced by others is shown
in Figures 70A-E. It can be seen from this alignment that there are some regions of
conservation in this class of proteins, suggesting that they are related in function, and
possibly in structure. Regions of similarity at the amino acid sequence level can be
used to design primers for *in vitro* amplification (PCR) by a process of back
translating the amino acid sequence to the possible nucleic acid sequences, then
choosing primers with the fewest possible variations within the sequences. These can
be used in low stringency PCR to search for related DNA sequences. This approach
permits the amplification of DNA encoding a FEN-1 nuclease without advance
knowledge of the actual DNA sequence.

20 It can also be seen from this alignment that there are regions in the sequences
that are not completely conserved. The degree of difference observed suggests that the
proteins may have subtle or distinct differences in substrate specificity. In other words,
they may have different levels of cleavage activity on the cleavage structures of the
present invention. When a particular structure is cleaved at a higher rate than the
25 others, this is referred to a preferred substrate, while a structure that is cleaved slowly
is considered a less preferred substrate. The designation of preferred or less preferred
substrates in this context is not intended to be a limitation of the present invention. It
is contemplated that some embodiments the present invention will make use of the
interactions of an enzyme with a less preferred substrate. Candidate enzymes are

tested for suitability in the cleavage assays of the present invention using the assays described below.

1. Structure Specific Nuclease Assay

Testing candidate nucleases for structure-specific activities in these assays is done in much the same way as described for testing modified DNA polymerases in Example 2, but with the use of a different library of model structures. In addition to assessing the enzyme performance in primer-independent and primer-directed cleavage, a set of synthetic hairpins are used to examine the length of duplex downstream of the cleavage site preferred by the enzyme.

The FEN-1 and XPG 5' nucleases used in the present invention must be tested for activity in the assays in which they are intended to be used, including but not limited to the InvaderTM-directed cleavage detection assay of the present invention and the CFLP[®] method of characterizing nucleic acids (the CFLP[®] method is described in co-pending Application Serial Nos. 08/337,164, 08/402,601, 08/484,956 and 08/520,946; the disclosures of these applications are incorporated herein by reference). The InvaderTM assay uses a mode of cleavage that has been termed "primer directed" of "primer dependent" to reflect the influence of the an oligonucleotide hybridized to the target nucleic acid upstream of the cleavage site. In contrast, the CFLP[®] reaction is based on the cleavage of folded structure, or hairpins, within the target nucleic acid, in the absence of any hybridized oligonucleotide. The tests described herein are not intended to be limited to the analysis of nucleases with any particular site of cleavage or mode of recognition of substrate structures. It is contemplated that enzymes may be described as 3' nucleases, utilizing the 3' end as a reference point to recognize structures, or may have a yet a different mode of recognition. Further, the use of the term 5' nucleases is not intended to limit consideration to enzymes that cleave the cleavage structures at any particular site. It refers to a general class of enzymes that require some reference or access to a 5' end to effect cleavage of a structure.

A set of model cleavage structures have been created to allow the cleavage ability of unknown enzymes on such structures to be assessed. Each of the model

structures is constructed of one or more synthetic oligonucleotides made by standard DNA synthesis chemistry. Examples of such synthetic model substrate structures are shown in Figures 30 and 70. These are intended only to represent the general folded configuration desirable is such test structures. While a sequence that would assume such a structure is indicated in the figures, there are numerous other sequence arrangements of nucleotides that would be expected to fold in such ways. The essential features to be designed into a set of oligonucleotides to perform the tests described herein are the presence or absence of a sufficiently long 3' arm to allow hybridization of an additional nucleic acid to test cleavage in a "primer-directed" mode, and the length of the duplex region. In the set depicted in Figure 71, the duplex lengths of the S-33 and the 11-8-0 structures are 12 and 8 basepairs, respectively. This difference in length in the test molecules facilitates detection of discrimination by the candidate nuclease between longer and shorter duplexes. Additions to this series expanding the range of duplex molecules presented to the enzymes, both shorter and longer, may be used. The use of a stabilizing DNA tetraloop [Antao *et al.* (1991) *Nucl. Acids Res.* 19:5901] or triloop [Hiraro *et al.* (1994) *Nuc. Acids Res.* 22:576] at the closed end of the duplex helps ensure formation of the expected structure by the oligonucleotide.

The model substrate for testing primer directed cleavage, the "S-60 hairpin" (SEQ ID NO:40) is described in Example 11. In the absence of a primer this hairpin is usually cleaved to release 5' arm fragments of 18 and 19 nucleotides length. An oligonucleotide, termed P-14 (5'-CGAGAGACCACGCT-3'), that extends to the base of the duplex when hybridized to the 3' arm of the S-60 hairpin gives cleavage products of the same size, but at a higher rate of cleavage.

To test invasive cleavage a different primer is used, termed P-15 (5'-CGAGAGACCACGCTG-3'). In a successful invasive cleavage the presence of this primer shifts the site of cleavage of S-60 into the duplex region, usually releasing products of 21 and 22 nucleotides length.

The S-60 hairpin may also be used to test the effects of modifications of the cleavage structure on either primer-directed or invasive cleavage. Such modifications